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An opsonised electrode

The direct electrochemical detection of superoxide generated by human neutrophils

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A pyrolytic graphite electrode was surface modified with human IgG and used as a stimulus to elicit a respiratory burst from human neutrophils. The oxidation current observed was shown to be due to re-oxidation of superoxide produced by the neutrophils. Both superoxide dismutase and *N*-ethylmaleimide were effective inhibitors of the oxidation current.

Superoxide *Human neutrophil* *Electrochemical detection* N-*Ethylmaleimide*

1. INTRODUCTION

Human neutrophils play an important role in the host defence mechanism. The generation of some cytotoxic agents by neutrophils is throught to proceed via reduction of dioxygen to superoxide, catalysed by an NADPH oxidase [1]. The evidence for superoxide production by stimulated cells includes cytochrome *c* reduction [2], nitroblue tetrazolium reduction [3], and spin trapping [4]. The superoxide thus formed may then react further to produce H_2O_2 and $OH^{\cdot}$, both potential cytotoxic agents. Neutrophils only generate these oxygen-derived metabolites as a response to certain stimuli. This is accompanied by a marked increase in dioxygen uptake known as the respiratory burst. It is believed that the stimulation of neutrophils involves a membrane perturbation. This may be effected by a soluble stimulus such as phorbol myristate acetate [5] or a surface coated with an immunoglobulin, e.g., IgG. We report here the stimulation of neutrophils at an electrode surface coated with IgG. We have used the electrode to oxidise superoxide produced by the neutrophils and have thus detected directly the primary cell metabolite of the respiratory burst.

2. EXPERIMENTAL

The electrochemical cell was water jacketed and thermostatted at 37°C, and was of a conventional 3-electrode configuration. A side-arm with frit contained the platinum gauze counter electrode, and a saturated calomel reference electrode (SCE, Radiometer K401) in a second side-arm was connected to the working compartment by a Luggin capillary. The working electrode, a 6 mm diameter pyrolytic graphite disc, was opsonised by dipping into a solution of human immunoglobulin (30 mg/cm^3 IgG, Miles Biochemicals) for approx. 1 min. The electrode was then washed thoroughly with distilled water and connected to the cell. It was rotated at 10.0 Hz to induce gentle hydrodynamic mixing. The electrode was poised at a potential of +50 mV *vs* SCE. This potential was considered sufficiently positive to oxidise superoxide, being >120 mV anodic of the accepted value for the standard potential, $E^{\ominus}(O_2/O_2^-) = -86$ mV *vs* SCE [6]. The experimental medium was RPMI 1640 (Flow Laboratories). The motor drive, motor speed controller, and potentiostat were from Oxford Electrodes. The current *vs* time traces were recorded on a Bryans 26000 X-Y recorder operating in the time-base mode.

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Human neutrophils were prepared by a modification of the method in [7]. Fresh venous blood from volunteers was collected into syringes containing 10 units/cm^3 heparin (Flow Laboratories). Dextran T-500 (Pharmacia) was added to a final concentration of 1% to help sediment erythrocytes. After 45 min the lymphocyte-rich supernatant was layered on top of a Ficoll-Paque solution (Pharmacia) and centrifuged for 20 min at $400 \times g$. Contaminating erythrocytes were removed from the cell pellet by hypotonic lysis for 30 s followed by adjustment to isotonicity using a 1.8% solution of NaCl. Further centrifugation at $400 \times g$ for 5 min yielded a cell pellet containing approx. 95% viable neutrophils. The cells were resuspended in RPMI 1640. Cells were counted in a Neubauer chamber after a 10-fold dilution of stock suspension into a solution containing 1% Crystal Violet and 1% acetic acid.

Oxygen consumption experiments were performed using a Clark type electrode (Rank Bros., Botisham, England). The stimuli used were polystyrene beads, mean diameter 0.75 μm (Polyscience) which had been incubated with IgG (15 mg·cm^{-3}) for 30 min. Each assay used 10^7 neutrophils in 0.5 cm^3 RPMI 1640 buffer. An injection of 0.1 cm^3 of a 1.25% (w/v) suspension of preincubated polystyrene beads was then used to elicit a respiratory burst.

3. RESULTS AND DISCUSSION

We have observed a burst of oxidation current at an IgG-treated pyrolytic graphite electrode when human neutrophils are added to the medium. This is shown in fig. 1A. After a short time lag of 30–40 s, the current rises to a plateau and then falls away. The plateau current observed is dependent on the number of cells added, as shown in table 1. The current shows little dependence on the rotation speed of the electrode, w; an electro-active species generated freely in solution and transported to a rotating disc electrode would lead to a current directly proportional to $w^{1/2}$ [8]. Both observations suggest strong adsorption of neutrophils to the surface of the electrode. This is consistent with recognition by the neutrophils of IgG on the electrode surface, and their concomitant binding to it. When a clean electrode, i.e., one not treated with IgG, is placed in the medium, subsequent addition

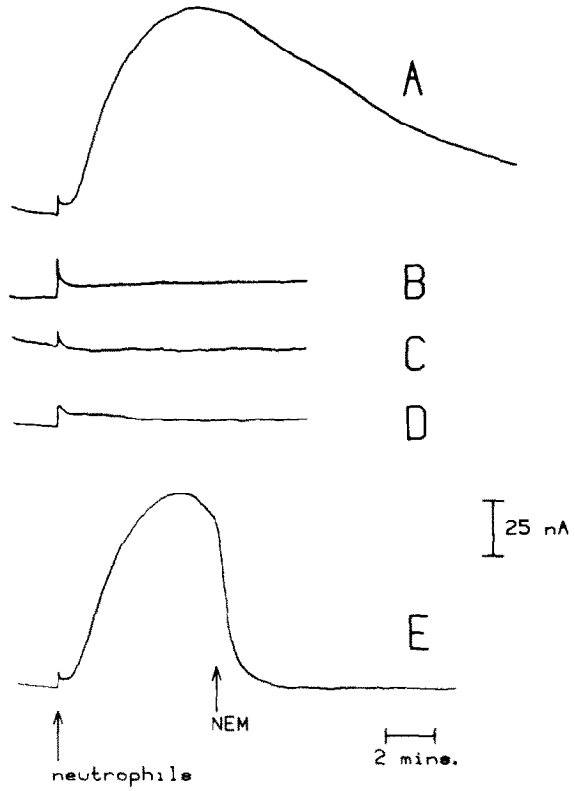

Fig.1. Current *vs* time traces at a pyrolytic graphite electrode in RPMI 1640 medium with 5×10^7 neutrophils added as indicated. (A) IgG-treated electrode; (B) clean electrode; (C) BSA-treated electrode; (D) IgG-treated electrode, medium containing 140 μm NEM; (E) IgG-treated electrode, with addition of NEM to 140 μm as shown.

of neutrophils produces no current response (fig.1B). Furthermore, treatment of the electrode with bovine serum albumin (BSA) (fig.1C) also fails to elicit any response. The mode of attachment of protein to the electrode surface is not well-

Table 1

Plateau current as a function of the number of neutrophils per unit volume

Cells $\times 10^6$/cm^3	Current (nA)
0	0
1.4	10.0
7.1	67.5
14.3	87.5

defined. Similar results observed on a gold electrode suggest a non-specific adsorption. These observations are consistent with neutrophils becoming attached to the IgG-coated electrode and being stimulated by it to produce a species oxidisable at the electrode. We believe this to be the superoxide anion.

The effect of added superoxide dismutase (SOD) was studied in order to investigate the identity of the electro-active species. Fig.2 (traces A–C) shows the respective plateau currents obtained for experiments in which 1.5×10^7 cells were added to medium (A), medium + superoxide dismutase (B), and medium + catalase (C). The oxidation current is decreased by >90% in the presence of $70\,\mu g \cdot cm^{-3}$ human CuZn SOD. This strongly suggests that the observed current is indeed due to direct electrochemical oxidation of superoxide. The lack of effect of catalase (fig.3C) rules out peroxide as the detected species. Injection of superoxide dismutase (SOD) into the medium during a respiratory burst

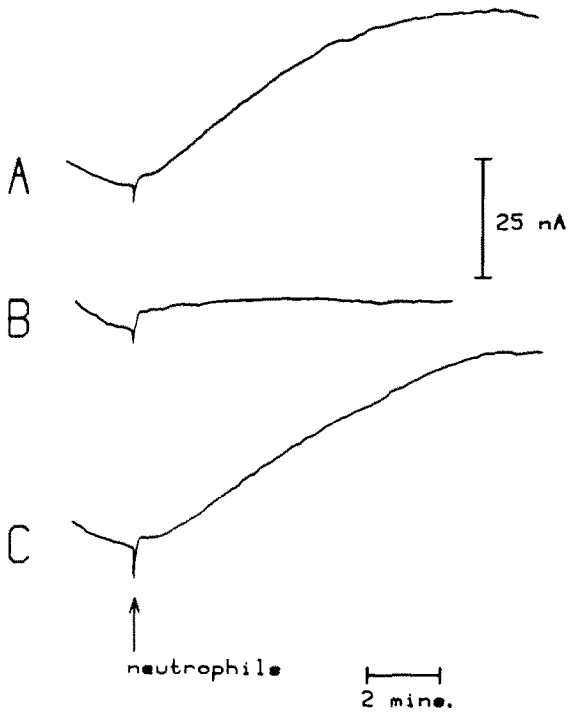

Fig.2. (A) Current *vs* time trace at IgG-treated pyrolytic graphite electrode in RPMI 1640 medium with 1.5×10^7 neutrophils added as indicated. (B) $+70\,\mu g/cm^3$ human CuZn SOD. (C) $+70\,\mu g/cm^3$ catalase.

produces less than 10% inhibition of oxidation current. This is consistent with the proposed strong adsorption of neutrophils onto the electrode surface which would lead to the superoxide produced being largely inaccessible to the added SOD.

Neutrophils can be irreversibly inhibited by the sulphydryl reagent *N*-ethylmaleimide (NEM) [9]. If NEM is present in the medium before neutrophils are added, no oxidation current is observed (fig.1D). However, if NEM is injected into the medium (fig.1E) during the respiratory burst, the oxidation current rapidly falls away to zero. The sulphydryl reagent is presumed to block metabolic pathways in the neutrophils. These may include the inhibition of NADPH oxidase which is ultimately responsible for the production of O_2^-. Isolated NADPH oxidase has been shown to be inhibited by the sulphydryl reagent *p*-chloromercuribenzoate [10]. The inhibition of the electrochemical oxidative burst is consistent with this explanation.

It is possible to make a comparison of the magnitude of the electrochemically observed current in the rotating disc experiment with the rate of oxygen consumption measured using the Clark electrode. If a neutrophil has a diameter of approx. $10\,\mu m$ [11] and we allocate an electrode surface area of $10^{-10}\,m^2$ to each cell in a monolayer, then an electrode of 6 mm diameter would be 'covered' by 2.8×10^5 cells. The oxygen consumption of the cells used in fig.1A was measured as 1.52×10^{-15} mol O_2/min per cell. Superoxide is formed by a one-electron reduction of dioxygen. However, net oxygen consumption depends upon spontaneous dismutation of superoxide to produce H_2O_2 and regenerate dioxygen. If all superoxide formed dismutes to H_2O_2, then a factor of 2 should be incuded when converting oxygen consumption to electron output by neutrophils. The rate of electron supply is therefore $1.52 \times 10^{-15} \times 2 \times 96\,500/60 =$ 4.8 pA/cell. The maximum rate of electron supply to the whole electrode via superoxide is therefore $1.35\,\mu A$. Fig.1A,E shows a plateau current of $0.09\,\mu A$, approx. 7% of the theoretical maximum. Given that the number of surface sites for electrode: cell recognition may be limited, and that no allowance was made for spontaneous dismutation of O_2^- in the vicinity of the electrode, we consider that the magnitude of the observed current is acceptable when compared with the oxygen consumption results.

In conclusion, we have demonstrated the first electrochemical oxidation of superoxide in aqueous solution at neutral pH. The combination of detector and stimulus in the guise of the IgG-coated electrode is fundamental to our successful observations. Finally, we have electrochemically detected, in the most general terms, a redox-active cell metabolite: superoxide generated by stimulated human neutrophils.

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